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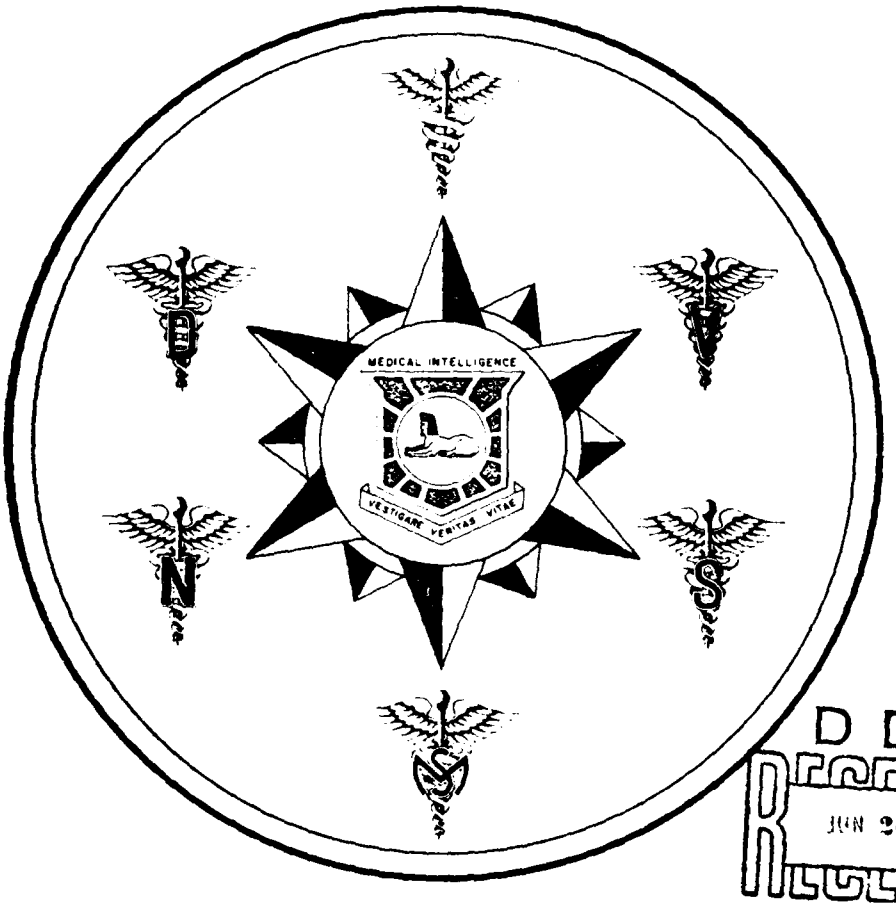

DEPARTMENT OF THE ARMY
OFFICE OF THE SURGEON GENERAL

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AN ELECTRON MICROSCOPY STUDY OF ADENOSINETRIPHOSPHATASE ACTIVITY OF STAPHYLOCOCCUS AUREUS

V. M. Kushnarev, A. S. Bykov
T. A. Smirnova and V. S. Tyurin

[Translated from *Mikrobiologiya*
Vol. 37, No. 1, 1968, pages 100 through 102]

The study of the association of various enzymes systems with structures of a bacterial cell has recently taken on great importance.

The majority of research on this question is associated with the appearance of dehydrogenases of certain gram-positive and gram-negative bacteria with the help of tellurite or tetrazole salts (Rosa and Tsou, 1963; Sedar and Burde, 1965; van Iterson and Leen, 1964). Phosphatase activity has been studied only in certain works (Voelz, 1964; Kushnarev and Smirnova, 1966 a,b; Done et al, 1965).

In the present report certain results of electron-microscopic research on the localization of the ATP-ase activity in *Staphylococcus aureus*, "Zhayev" strain are presented.

Methods

We have selected the "calcium" method of Padikul, German (Berston, 1965) for reviewing ATP-ase activity, since the "Lead" method of Gomor (Pirs, 1962) proved to be non-specific.

The staphylococcus culture was cultivated in Hottinger meat-peptone broth, pH 7.2-7.4 at 37°. . The sediment of bacteria was washed three times with physiological solution and suspended in 1-2 ml of barbital buffer pH 9.4. The bacteria were placed in centrifuge tubes with incubation medium for determining the ATP-ase which was preliminarily heated at 37° for 20 minutes. ATP was not added to the medium for the control. Incubation was carried out at 37° for 5-60 minutes. .

The incubation was terminated by fixation of the cells according to Riter et al (Riter et al, 1958). The bacteria sediments were dehydrated in alcohols, poured into methacrylate (butylmethacrylate: methylmethacrylate-4:1). The blocks were polymerized at 56° for 24-48 hours with benzoyl peroxide. Sections were obtained on an ultramicrotome LKB "Ultratom" 4800 and were contrasted with 5% aqueous solution of uranyl acetate for 30-60 minutes. A portion of the sections was not contrasted. The sections were examined in the electron microscope UEMV-100 at an accelerating voltage of 75 kv and with an instrumental magnification of 18,500X-40,000X. Photoplates-MR.

Results and Discussion

The structure of cells of *Staph. aureus* strain "Zhayev" was studied and described by us earlier (Pashkov et al, 1965). The cell wall consists of 3 or 5 layers of varying electron density, behind which are brighter intervals with transverse partitions (these do not always appear), going from the walls to the cytoplasmic membrane. The thickness of layers of cytoplasmic membrane of the cells are more or less equal. In a portion of the sections the inner layer of the cytoplasmic membrane does not appear.

In Figure 1-5, sections of cells of staphylococcus aureus are shown after incubation with ATP.

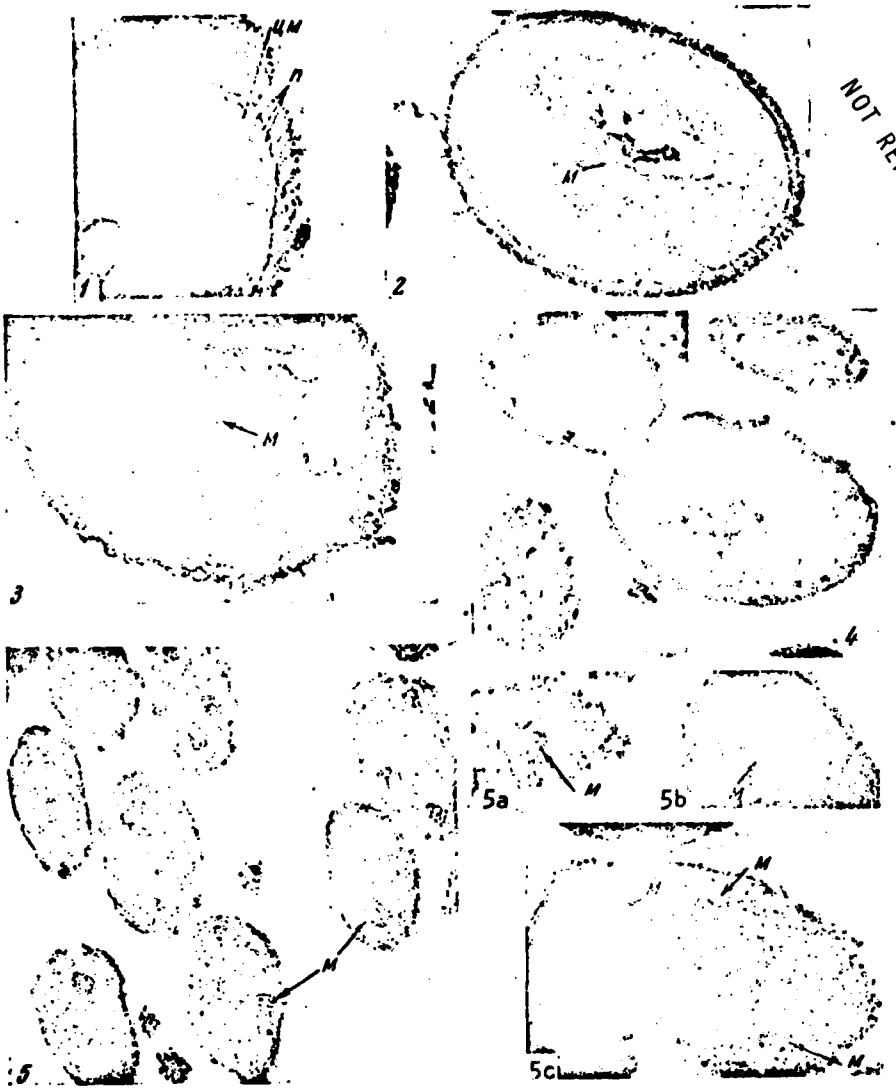

Figure 1. Sections of cells of Staph. aureus strain "Zhayev" after incubation with ATP for 30 minutes. The cytoplasmic membrane (CM), nucleoid (n), partitions (P) between the CM and the cell wall are clearly visible. 74,000X.
Figure 2. The same. 60,000X. Mesosome (M) contrasted with phosphate in the region of the nucleoid.
Figure 3. The same. 70,000X. Tubular mesosome, contrasted with calcium phosphate.
Figure 4. The same, but incubation with ATP for 10 minutes. Sections not contrasted with uranyl acetate. Deposits of calcium phosphate in the cell wall CM. 46,000X.
Figure 5. The same. 46,000X. Mesosome (M), contrasted with calcium phosphate. a, b, c -- fragments of Figure 5. 60,000X.

The calcium phosphate which forms as a result of incubation with ATP has sufficient contrast and appears in the form of electron-optically dense material, which in addition are in contrast to cellular structures, or is in the form of an inclusion.

The greatest depositing of calcium phosphate in the cells is observed during the first 30 minutes of incubation with ATP. In Figure 1 (incubation with ATP for 30 minutes) the structure of the cell wall is clearly visible. The cytoplasmic membrane is significantly thinner in places and acquires additional contrast as a result of impregnation with calcium phosphate. Considerably more often than in the control, in almost all sections transverse partitions of a regular character are visible. In places calcium phosphate forms electron-optically dense granules from 60 to 70 Å in size. The cytoplasmic membrane is a clearly different elementary membrane (Figure 1-2). Cytoplasmic material of a fine granular character in places forms portions with increased electron-optical density. These portions are located at different sites of the cytoplasm. In the cytoplasm and nuclear vacuole "holes" frequently appear, surrounded by a membrane which is considerably denser than in the control. These portions apparently correspond with mesosomes of the cell. In Figure 3 an intranuclearly located mesosome, contrasted with calcium phosphate, is visible.

After 10 minutes of incubation with ATP, in uncontrasted sections of the cell we observed certain types of deposits of calcium phosphate, namely: 1) circular inclusions, located under the cell wall, in the intercellular partitions, the thickness of the cell wall and in the cytoplasmic membrane (Figure 4); the inclusions were ~350 Å in size; 2) mesosomes contrasted with calcium phosphate and having various profiles, probably depending on the thickness of the section (Figure 5 a - c).

The mesosomes have the form of tubes sliced lengthwise or crosswise. The diameter of the tubes are on the order of 180-220 Å, , the length up to 1600 Å . The thickness of the electron-optically light portion of the tube is 148 Å (this part was not contrasted with calcium phosphate). A cross section of the dark portion is 80 Å .

The formation described cannot be related to artifacts, since in similar sections of controlled preparations they did not appear even after contrasting with uranyl acetate.

The distribution of deposits of the product of the reaction in the cell wall may be partially determined by the activity of the transport of ATP-ase (ATP-ases). The distribution of calcium phosphate in the mesosomes, cytoplasmic membrane and intercellular partitions is most rapid of all as a result of the activity of ATP-ase which participates in oxidative phosphorylation. One may also assume that localization of phosphate granules in a layer of the cell wall and on it is secondary, i.e., as a result of settling of insoluble granules of calcium phosphate from the cell. We observed similar phenomena in gram-negative bacteria (Kushnarev and Vysotskiy, 1966).

In contrast to the data of Suganuma (Suganuma, 1965), who considers that the cell wall of staphylococci has a triple-layered structure, our results indicate rather a five-layered structure of the cell wall, which corresponds with data of Pashkov et al, 1965. It is generally accepted to consider the cytoplasmic membrane as a three-layered structure. However, Suganuma (Suganuma, 1965) suggests that the third (external) layer of the cytoplasmic membrane in staphylococci is general for a cell wall and for a cytoplasmic membrane. In Figure 1 the five-layered cell wall and three-layered cytoplasmic membrane, separated from the wall of the electron-optically bright region, are clearly visible.

Conclusions

Electron-microscopic study of the localization of ATP-ase activity in unfixed cells of *Staph. aureus* strain "Zhayev" is possible by depositing calcium phosphate -- the primary product of the ATP-ase reaction.

ATP-ase activity in cells of *Staph. aureus* strain "Zhayev" is concentrated in the cytoplasmic membrane, mesosomes, intercellular walls.

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IDENTIFICATION OF A FACTOR IN STAPHYLOCOCCUS ALBUS EXTRACTS, WHICH CAUSES PENICILLINASE FORMATION IN STAPHYLOCOCCUS AUREOUS CELLS.

Ye Vyshepan and L. V. Meleshkina

[Translated from *Mikrobiologiya* (Microbiology), Vol. 37, No. 2, 1968, pp. 281-285.]

A factor in *Staphylococcus albus* extracts which causes regeneration of penicillinase in *Staph. aureus* cells has been studied. This factor, which is also present in a penicillinase extract may be concentrated by alcohol precipitation. Upon deproteinization of the extract by chloroform with isoamyl alcohol, the ratio of the factor to the penicillinase increases. Upon separation of a column of Sephadex G-200, the factor is eluted almost simultaneously with the penicillinase. The factor is resistant to treatment with RNase and DNase, but according to preliminary date is not resistant in the presence of trypsin. The assumption is made that the factor is messenger RNA of *Staph. albus*, associated with protein. Other possible explanation of the observed effect are also considered.

In previous work (Vyshepan and Meleshkina, 1967) we have shown that upon incubation of a suspension of *Staphylococcus aureus* in meat-peptone broth with certain pherol preparations of RNA or with salt extracts of *Staph. albus*, regeneration of penicillinase occurs.

On the basis of this fact the assumption was made as to the presence in the phenol preparations of RNA and in salt extracts of *Staph. albus* of a certain factor (presumably RNA), which causes the formation of the indicated enzyme. Since in phenol preparations of RNA and especially in extracts, both DNA and other contaminates may be present, there is no assurance that the factor causing regeneration of the enzyme *Staph. aureus* cells is just their RNA.

In this work we had attempted to isolate the factor which interests us from penicillinase and other proteins, and also have tested the effect of nucleases and trypsin on the activity of the factor.

Methods

The method of obtaining the *Staph. albus* extract and the suspension of *Staph. aureus*, and also the method of determining penicillinase activity and the activity of the factors studied have been described in our previous work (Vyshepan and Meleshkina, 1967). Here let us indicate only that by the activity of the factor we mean the ability of extracts of *Staph. albus* to increase penicillinase activity pre-existing in the extract upon incubation with a suspension of *Staph. aureus* in meat-peptone broth (MPB). The control is a sample in which the extract is incubated only with MPB. Penicillinase activity was expressed in milliliters of 0.02 NI_2, which is bound after incubation of the sample of penicillin.

In the given work these methods were used.

1. Concentration with alcohol.

The extract was concentrated by precipitation in the cold with three volumes of ethanol. The precipitate was collected by centrifugation and dissolved in a small amount of 1M NaCl.

2. Deproteinization.

The abstract was deproteinized in the cold with chloroform with 2.5% isoamyl alcohol and was further precipitated from the aqueous layer with ethonal, as described above.

3. Chromatography on Sephadex.

Chromatography of the extract was carried out on Sephadex G-200. In order to obtain a gel 2g of Sephadex was placed in 70 mlof 1 M NaCl. After two days the column was packed with the gel which formed. A column with diameter of 1cm and height of 50 cm was used. 0.8 ml of the concentrated extract was deposited on column. The rate of penetration through the column of a 1 M solution of NaCl was approximately equal to 4ml per 15-8 minutes. Fractions of about 4 ml, and sometimes of about 2 ml, were selected. In all fractions the extinction was determined at 260 and at 280 m as well as the activity of penicillinase and the activity of the factor.

Results and Their Discussion

The addition of ethonal to the abstract with the purpose of concentrating the factor caused precipitation both of the factor and also of penicillinase. In order to free it from the penicillinase, attempts were made to deproteinize the extract of chloroform. In this process it was found that a single treatment of the extract with chloroform lead to an increase in concentration of the factor and to a greater degree than concentration of the penicillinase. However, upon repeated treatments with chloroform both activities were lost.

In order to clarify the nature of the factor, experiments were carried out with chromatography of concentrate extracts on Sephadex. Data of two of these experiments are presented in figures 1 and 2 from which it can be seen that the factor which was responsible for the increase in penicillinase activity is eluted from the column simultaneously (Fig. 1) or somewhat earlier than the penicillinase (Fig. 2). Both the penicillinase and the factor begin to be observed in fractions following the small rise at 260 m The maximum separation will be in fractions preceding the large absorption peak at this wavelength. Absorption at 280 m was 1.7-2 times less than absorption at 260 m ; consequently the amount of protein in these fractions varied.

During chromatography the factor and the penicillinase were eluted from the column directly after the elution of hemoglobin (Molecular weight 68,000), which we used as the standard. These data indicate that the molecular weight of the factor and of the penicillinase are close to 60,000, if separation on Sephadex preceeds according to the principle of a molecular seive. Consequently, high molecular DNA cannot be of interest to us as the factor and the observed formation of penicillinase cannot be genotypic transformation of *Staph. aureus*.

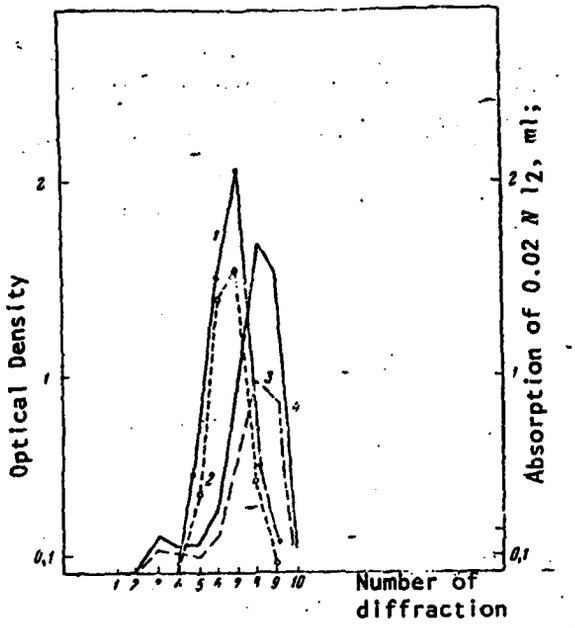
If the factor were RNA, the molecular weight of the protein whose formation it should code, should be at least six tons lower.

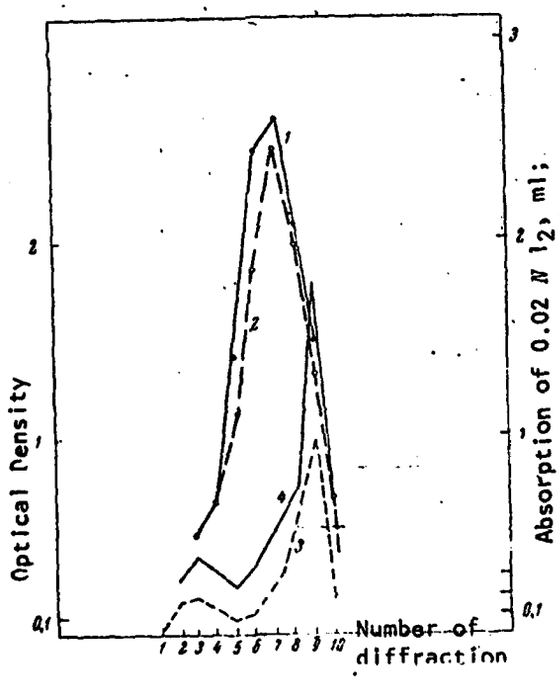
The data which we obtained may be explained either by the fact that the factor is not identical with mRNA or by the fact that in the extract there is a very close complex of mRNA with penicillinase, as a result of which the lighter penicillinase is eluted from the column along with the heavier RNA. At the beginning of the work (Vyshepan and Meleshkina, 1967) we assumed the possibility of the existence of such a complex both in the extracts and also in certain RNA preparations obtained according to Kerba, considering that such complexing of RNA may protect it from the effect of nucleuses of the recipient and determine its biological effect.

In 1963 Hoagland *et al.* (Hoagland *et al.*, 1963) observed that the biologically active fraction of cytoplasmatic RNA of liver is resistant to the effect of RNase and is sensitive to the effect of trypsin. The authors assume a complexing of RNA with protein, which they play an important role in the biological effect of this RNA.

As our experiment showed, treatment of extracts with RNase and DNase did not deprive the extract of its activity as the factor is free to stimulate an increase in penicillinase activity upon contact with *Staph. aureus* cells in MPB; after treatment with trypsin, according to our preliminary data, the extract lost this ability. We present one experiment of this series in the table for illustration.

It is interesting to note that all of these enzymes (and as we accidentally observed -- hemoglobin) upon incubation with the extract reduce penicillinase activity of the extract (compare samples 1 and 3, experiments 1-3). Moreover, the decrease in penicillinase activity in the presence of trypsin is not so great that it could be suspected of having hydrolyzed the peincillinase with this enzyme.





Figures 1 and 2 [Caption next page]

Figures 1 and 2 [caption]. Fractionation of Extract on Column with G-200 Sephadex. 1, Penicillinase activity after incubation of 0.6 ml of fraction with 1.5 ml of 4-hr suspension of *St. aureus* in MPB; 2, Penicillinase activity after incubation of 0.6 ml of fraction with 1.5 ml of MPB; 3, Absorption at 280 mμ; 4, Absorption at 260 mμ

Saz and coauthors observed inhibition of staphylococcal penicillinase by certain dipeptides (Saz et al., 1961). It is possible that proteins may also exhibit such an effect.

From these experiments it follows that the presence of RNase and DNase at the time of contact of the factor with *Staph. aureus* cells does not suppress the ability of these cells to form penicillinase. Moreover, in all experiments with RNase (five experiments in all) we noted a larger increase in penicillinase activity than in similar experiments without RNase (Table, experiment 1: 0.96 and 1.26). It is possible that the presence of RNase, which destroys unprotected RNA of the recipient, alters the competition between RNA of the extract and RNA of the recipient in favor of RNA of the extract at sites of protein synthesis.

As for trypsin, the suppression of penicillinase formation may be caused (or complemented) not only by its effect on the factor, but also by its effect on *Staph. aureus* cells, since it was not eliminated, and the medium was only acidified to pH 6.5.

Since the factor is apparently associated with protein and its resistance to RNase, we now see two possible explanations of the facts which we have observed.

It is possible that the factor is *Staph. albus* RNA, well protected by protein from the effect of RNase and from nuclease of the recipient. Treatment with trypsin may destroy the protein which protects the *Staph. albus* RNA and thus make the RNA accessible to the effect of nucleases. seems extremely probable to us that the protective RNA may be present in very low quantities in various preparations of "pure" RNA and may determine the biological activity of RNA hydrolyzates as well.

The second possible explanation for our observations leads to the less probable hypothesis that all *Staph. aureus* strains are inducible in relation to penicillinase, but that the inducer for formation of this enzyme is not penicillin, but some other substance present in the extracts

and in *Staph. albus* RNA preparations. This position cannot be eliminated, considering the data of Saz and Lovery (Saz and Lovery, 1964), which show that the inducers of penicillinase formation in certain staphylococci may be substances very different from penicillin in chemical nature, for example cyclic peptides, homogramicidin or dimethyl oxyphenyl.

Effect of RNase, DNase, and Trypsin on the Activity of the Factor in the Extracts

Experiment Number	Sample Composition During Preincubation, 60 min at 37	Sample No.	Composition of Sample During Determination of Biological Activity	Absorption of 0.02 Nl_2, ml in Sample
1	Extract Diluted 1:1 with Phosphate Buffer 0.1 M pH 7.00 -- (C_1)	1	0,6 ml C_1 + 1,5 ml MPB	4,91
		2	0,6 ml C_1 + 1,5 ml Staph. aureus in MPB ~$5 \cdot 10^8$ organisms/ml	5,87
	The Same, but 100 µ g/ml RNase in Buffer (C_2)	3	0,6 ml C_2 + 1,5 ml MPB	2,87
		4	0,6 ml C_2 + 1,5 ml Staph. aureus in MPB ~$5 \cdot 10_8$ organisms/ml	4,13
2	Extract Diluted 1:1 with Phosphate Buffer 0.2 M pH 6.5 with 10^{-2} M Mg-acetate (C_1)	1	0,6 ml C_1 + 1,5 ml MPB	5,65
		2	0,6 ml C_1 + 1,5 Staph. aureus in MPB ~$5 \cdot 10^8$ organisms/ml	5,97
	The Same, but 100 g/ml /DNase in Buffer (C_2)	3	0,6 ml C_2 + 1,5 ml MPB	2,79
		4	0,6 ml C_2 + 1,5 ml Steph. aureus in MPB ~$5 \cdot 10^8$ organisms/ml	3,26
3	Extract Diluted 1:1 with 0.02 M Buffer pH 8.0, After Preincubation Mixture was Diluted 1 1/2 times with 0.2 M Phosphate Buffer pH 6.5 (C_1)	1	0,6 ml C_1 + 1,5 ml MPB	3,70
		2	0,6 ml C_1 + 1,5ml Staph. aureus in MPB ~$5 \cdot 10^8$ organisms/ml	4,26
	The Same, but 100 g/ml trypsin in Buffer During Preincubation (C_2)	3	0,6 ml C_2 + 1,5 ml MPB	3,43
		4	0,6 ml C_2 + 1,5 ml Staph. aureus in MPB ~$5 \cdot 10^8$ organisms/ml	2,55

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CHANGES OF STAPHYLOCOCCUS ULTRASTRUCTURE UNDER THE EFFECT OF ANTIBIOTICS OF THE PENICILLIN GROUP AND 6-AMINOPENICILLANIC ACID

L. N. Kats, A. F. Mooz, and E. A. Bayntraub

[Translated from *Mikrobiologiya* (Microbiology)
Vol. 37, No. 1, pp. 95-99, 1968

and also after 1-time action subinhibiting and higher concentrations of the antibiotics on the initial and antibiotic-resistant variants. The cells were fixed with osmium fixative of Rider and Kellenberger (standard formula), they were dehydrated in alcohols and embedded in methacrylate. Ultrathin sections were obtained on a LKB-Produkter ultratome and were stained with uranyl acetate. Preparations were studied and photographed in an electron-microscope JEM-7 at an accelerating voltage of 80 kv and with instrumental magnification of ×35,000 and ×50,000.

Results

In the original srain of staphylococcus, not subjected to the effect of antibiotics, the cells have a spherical or oval form, ~1μ in size. The cell wall is formed from a homogeneous electron-dense material and has a total thickness of 250-300 Å (Figure 1, 2, 3). The inside layer of the cell wall differs from the remaining part of the cell wall by the high electron-optical density- its thickness is 80-75 Å. The cytoplasmatic membrane usually appears as single-circuit, and only at sites of cross-sectional division of the cell can one sometimes observe a double-circuit cytoplasmatic membrane (total thickness ~75Å). In lysed cells of staphylococcus the double-circuit structure of the cytoplasmatic membrane is the rule. The cytoplasm is so densely filled with a granular component, that individual ribosomes are not always clear. In the cytoplasm are well developed, complexly structured membrane structures, in which the membrane component is usually masked by a material equal in thickness and the electron has an optical density to the material of the inner layer of the cell wall. The membrane structures are not infrequently associated with the ends of newly forming cross-sectional walls (Figure 1, 3). The latter are formed during cell division and grow centripetally from two opposite sides of the cell. The cross-sectional walls are bordered on two sides with material of the inner layer of cell wall and contain this material in the middle portion as well (Figure 2, 3). Nuclear material occupies a considerable part of the cytoplasm and is filled thickly with the fibrillar material of DNA.

Under the influence of antibiotics on the submicroscopic structure of the cell distinct changes occur which are basically identical for all antibiotics studied.

After a single action of antibiotics on the original strain of *Staph. aureus* (Figures 4-7) the cells had an oval or imperfectly elongated form and were larger than in the control. The cell wall is usually thicker (100-150 Å), in comparison with the original culture,

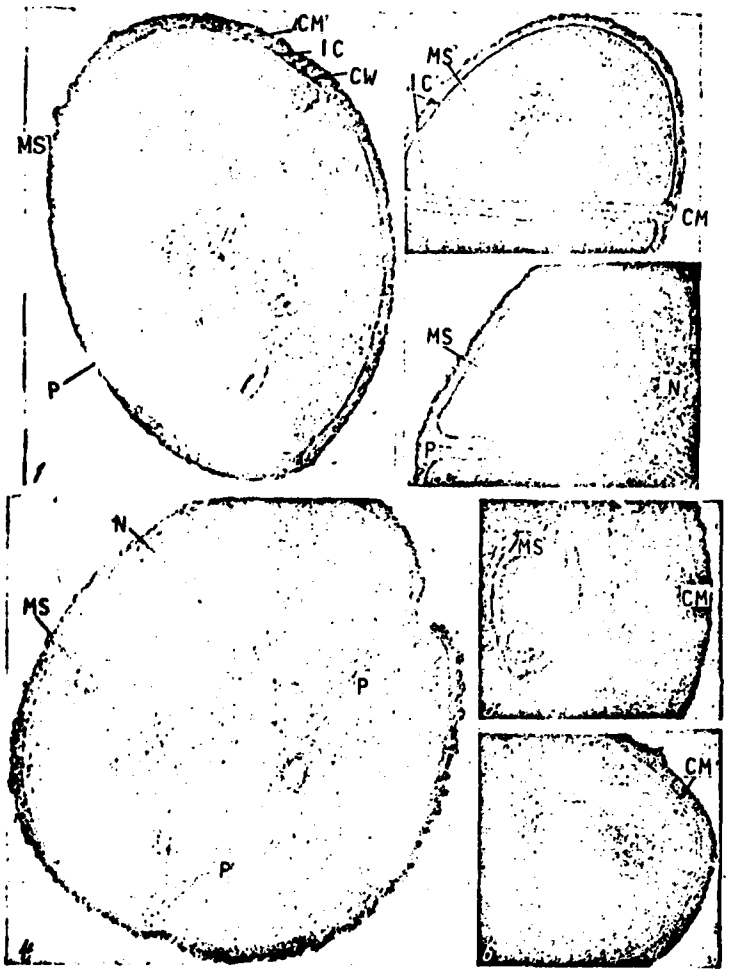

Figure 1, 2, 3. Original strain of *Staph. aureus* (control). 100,000X, 85,000X, 120,000X respectively.

Figure 4. Variant obtained from original strain after single action of ampicillin 112,000X.

Figure 5, 6. Variant obtained from original strain after single action of penicillin. 130,000X, 112,000X respectively.

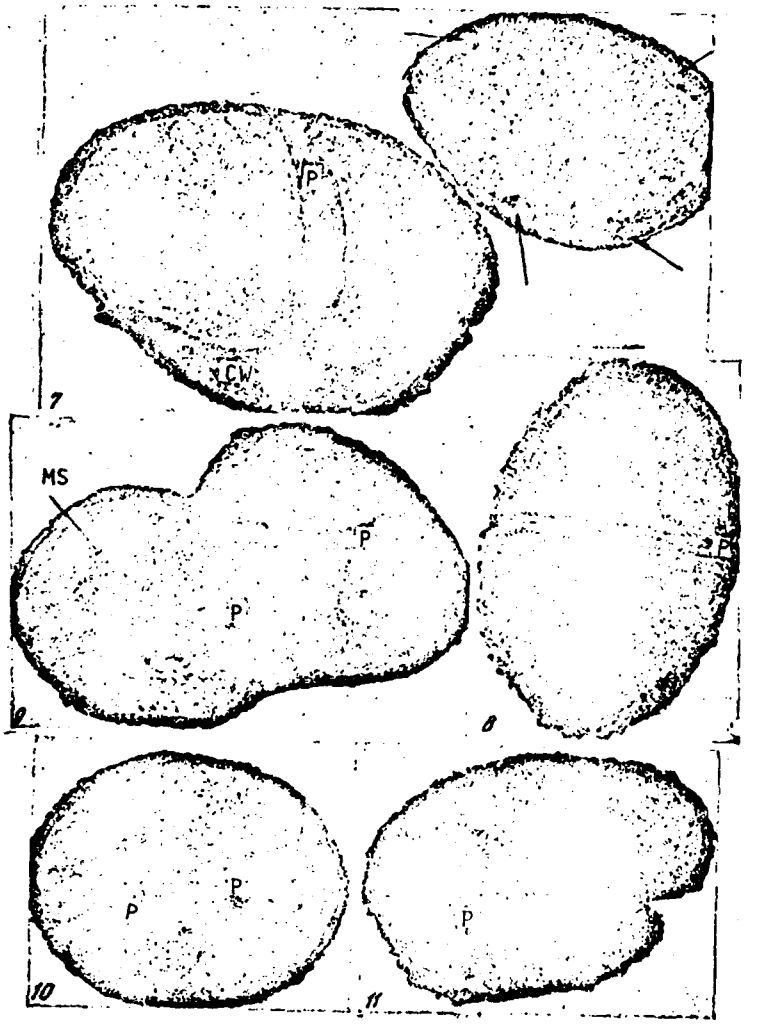

Figure 7. Variant obtained from original strain after single action of 6-APA. 90,000X

Figure 8. Variant resistant to penicillin, obtained from original strain after multiple receding with increasing concentrations of this antibiotic. 135,000X

Figures 9, 10, 11. Variant obtained after single action of ampicillin in ampicillin-resistant variant. 72,000X 61,000X, 61,000X respectively.

Conventional symbols for Figures 1-11:
CW-cell wall; IC-inner layer of cell wall; CM-cytoplasmic membrane; MS-intracytoplasmic membrane structures; N-nucleoid; P-partitions forming during cell division.

while its thickness is not identical in various portions of the cell. Sometimes in the cell wall irregular cleavage is observed (Figure 7, arrow) or inclusion of material with a low electron-optical density (Figure 7, arrows). Material of the inner electron-dense layer of the cell wall which appears clearly in the original culture is partially (Figure 4, 6) or completely (Figure 5, 7) reduced. In contrast to the original strain, it also almost completely disappears from transverse walls and from the cytoplasmatic membrane structures (especially under the influence of ampicillin and 6-APA, Figures 4, 7). The cytoplasmatic membrane usually appears as a double circuit (Figure 5), while its disrupted osmiophyllic layer is associated from the cell wall with transverse connectors (Figure 6). The content of the cytoplasm possesses less electron-optical density than in the control, but individual ribosomes are almost not visible (Figure 4, 6). Membrane structures in the cytoplasm are usually less developed than in the original strain and, as a rule, are not associated with transverse walls (Figure 5). Sometimes they have a tubular structure; in cross-sectional slices the tubes consist of two rings inside each other (Figure 6). Such tubular membrane structure arose mainly under the influence of penicillin and were not encountered in the original culture. It is interesting that usually they are localized in the peripheral part of the cytoplasm near the cell wall. Nuclear material is impregnated in the cytoplasm with small particles and occupies a small part of it in comparison with the control (Figure 4). Transverse walls are often sharply thickened and form irregularly in different directions, while their formation is usually not accomplished by the separation of daughter cells from each other. As a result of this large cells are formed which are complexly broken up by transverse walls (Figure 4).

Variants which are resistant to antibiotics differ considerably less in their submicroscopic structure from the original strain than cultures obtained after single action of antibiotics on the original strain (Figure 8). Cells of resistant variants usually have true oval or rounded forms, but are smaller than in the control (0.5-0.6μ). The cell wall is regularly thickened in different parts, and its internal electron-optically dense layer is poorer than in the original culture, but is never completely reduced. The cytoplasmic membrane is usually a single-circuit, but is joined to the cell wall by thin connectors, which are not observed in the original culture. The membrane structures in the cytoplasm are less developed than in the original culture, but partially contain material of the inner layer of the cell wall, which is characteristic for the original strain. Not infrequently they are surrounded by vacuoles which apparently form at the site of inclusion, which are destructive during production of preparations. The nuclear material is included in the cytoplasm as small particles which do not appear at all. The transverse partitions are bordered on two sides

with material of the internal layer of the cell wall. Their formation is accomplished by division of two daughter cells, as occurs in the original culture (Figure 8). Only in the methicillin-resistant variant does the internal electron-dense layer of the cell wall almost completely disappear, as normal cell division is sharply disrupted.

During action of subinhibiting and higher concentrations of penicillin preparations on resistant variants of *Staph. aureus*, changes in the submicroscopic structure of the cells are observed which are as pronounced as follows after single action of antibiotics from the original strain (Figures 9, 10, 11). These changes are expressed in a marked increase in the dimensions of the cell, thinning of the cell wall, elimination of its inner electron-dense layer, in reduction of the amount of membrane structures in the cytoplasm and emergence of anomalous transverse partitions, and also disruption of normal cell division.

Discussion

As the present research has shown, the morphological consequences of action of various antibiotics of the penicillin group (penicillin, ampicillin, methicillin, 6-APA) on the ultra-structure of staphylococcus are basically similar. In the literature there are also indications of the similarity of the chemical effect of penicillin and methicillin on gram-positive bacteria (proper name Ciak and Hahn, 1962; Warren and Gray, 1963; Aldrich and Sword, 1964), and also of the similarity of those morphological changes which these antibiotics cause (Fitz-James and Hancock, 1965).

In staphylococcus these changes are expressed in shedding of the inner electron-dense layer, adjacent to the cytoplasmic membrane, in the appearance of anomalous partitions and disorganization of cell division, which is not completed by the separation of the daughter cells which have formed, in the appearance of double-circuit cytomplasmic membrane instead of single-circuit one and rearrangement of the intra-cytoplasmic membrane structures, and also in certain rudimentary nuclear material.

At the present time it is difficult to say with certainty which of these changes are the result of the direct action of the antibiotic and which arise secondarily. However, comparison of cytoplasmic changes observed with biochemical studies makes it possible to hypothesize that disruption of the structure of the cell wall and also disorganization in the formation of transverse partitions and normal cell division are the direct result of the action of the penicillins. Thus, cytological data which indicates that under the influence of penicillin the cell wall becomes thinner in staphylococcus and the inner electron-dense

layer disappears (Murray et al., 1959; Suganuma, 1962; Pashkov et al., 1965), are in good agreement with biochemical data on the disruption of mucopeptide synthesis in these bacteria (Park and Strominger, 1957; Perkins, 1963). Finally, cytological data on the development of the anomalous transverse partitions, which often occupy a great part of the cell (Suganuma, 1962; Murray et al., 1959), also agree with biochemical data on the accumulation of precursors of polysaccharide components of the cell wall in the cell (Park and Strominger, 1957; Ciak and Hahn, 1962; Warren and Gray, 1963).

The indicated changes in the structure of the cell wall and in the nature of deformation of transverse partitions are certainly accompanied by changes of the membrane apparatus of the cell. Thus, under the influence of penicillins the double-circuit structure of the cytoplasmic membrane regularly appears instead of the single-circuit one which is characteristic for cells not subjected to the effect of the antibiotics. It is interesting that the indicated change in the structure of the cytoplasmic membrane was observed after natural autolysis of the cells (Suganuma, 1965; Avakyan and Kats, 1967), and also after hydrolysis in one *N HCl* (Suganuma, 1966). It is possible that in all cases partial disruption of the granular component of the cytoplasm occurs as a result of which the inner layers of the cytoplasmic membrane, previously masked by granules of cytoplasm, become clearly visible (Suganuma, 1966).

Under the influence of penicillin antibiotics the nature of the intracytoplasmic membrane structures also changes. Thus, in a normal culture staphylococcus, not subjected to the action of the antibiotic, there are three types of membrane structures which differ not only in morphology, but apparently also in function (Suganuma, 1962; Kats, 1966; Avakyan and Kats, 1967). One of these three types of membrane structures is associated with the formation of transverse partitions. Under the influence of penicillin and ampicillin the membrane structures of the latter type disappear, and cell wall material is not contained in the available membrane structures. Along with this the so-called tubular membrane structures arise, which are usually not encountered in normal culture (our observations). The selective appearance of such membrane structures under the influence of antibiotics and their peripheral position in the cell enables one to conclude that they fulfill an excretory function. Similar indications on the possibility of excretory functions of membrane structures in bacteria also appear in the literature (Tomasz et al., 1964; Iterson van, 1965). Thus, various membrane structures in the same bacterial cell may have different functions (Suganuma, 1965; Voelz, 1965; Cohen-Bazire et al., 1966; Avakyan and Kats, 1967).

Conclusion

In ultra-thin sections the effect of single action of suninhibiting and higher concentrations of penicillin, methicillin, ampicillin, and 6-aminopenicillanic acid has been studied on the structure of the original strain *Staph. aureus*, which is sensitive to these antibiotics, and the effect of the same antibiotics of the structure of antibiotic-resistant variants obtained from the original strain. The morphological consequences of the effect of the indicated antibiotics were basically similar. They are expressed in thinning of the cell wall and disappearance of its inner electron-dense layer, adjacent to the cytoplasmic membrane. Moreover, they are expressed in an appearance of anomalous partitions and disorganization of cell division, and the appearance of a two-circuit cytoplasmic membrane in place of the single-circuit one and reorganization of the intracytoplasmic membrane structures, and also in certain rudimentary nuclear material. Under the influence of penicillin, tubular membrane structures arise which apparently fulfill an excretory function.

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CONCERNING THE CHANGES OF SOME COAGULATION FACTORS IN PRESERVED BLOOD

P. A. Ryaby

Coagulation factors were examined in preserved blood with various storage periods (from 1 to 30 days). It was noted that the activity of the majority of coagulation factors in preserved blood fell with increased storage period; as to fibrinolytic activity--it increased.

Transfusion of blood and its components continues to remain the main method of therapy for hemorrhagic diatheses and various hemorrhages. Therefore, determination of the retention of coagulation factors in preserved blood in the process of its storage is of real significance.

For complete hemostatic effect, transfused blood should contain the maximum of substances of a protein nature which participate in the coagulation mechanism. In the process of storage of preserved blood, complex morphological and physiochemical changes occur, whose nature and depth depend both on the composition of the preservative and also on the conditions and length of storage. All of this affects the coagulation factors in the preserved blood.

M. K. Deval'd shows a progressive reduction in the level of prothrombin in preserved blood. Thus, according to data of the author, after 10 days it contains 95% of the original amount of prothrombin, and by the twentieth day of storage--76%. F. R. Vinograd-Finkel' found prothrombin in preserved blood on the fortieth day of storage. R. M. Glants did not notice any regularity of fluctuation of the level of prothrombin in preserved blood. V. L. Lemenev believed that the concentration of this coagulation factor during the first days of storage of blood even increases under certain conditions. According to data of A. P. Kuchek, the amount of prothrombin during storage of preserved blood remains stable.

G. L. Rubinshteyn and M. P. Petrova, Cousin et al assert on the basis of multiple experiments that after a week the retraction ability of a blood clot is noticeably reduced, and during a more prolonged period of storage retraction is practically absent. V. I. Teodorovich reports a two-fold decrease of retraction by the end of five days of storage of preserved blood. A. A. Markosyan considers that for the beginning and continuation of the retraction process, not only the presence of a specific amount of thrombocytes, but also the integrity of their morphological structure are important, and as is known, it is considerably disrupted during the first days of storage of blood. Mustard has noted that towards the end of the third week about 16-41% of intact thrombocytes are still retained in preserved blood. R. A. Rutzberg and G. M. Abdullayev, P. M. Al'perin and L. A. Zherebtsov consider that thrombocytes in preserved blood are easily agglutinated and rapidly destroyed. According to data of V. L. Lemenev, destruction of thrombocytes begins at the moment of taking of the blood, and after a day a noticeable reduction in their amount occurs.

Janiakowa et al has established that thromboplastic activity of plasma of freshly preserved blood constitutes 80% of the activity of blood of donors and over the course of four weeks of storage it changes slightly. As regards the time of reclassification of plasma, according to data of F. R. Vinograd-Finkel', and also of A. P. Kuchek, it extends in parallel to the periods of time of preservation of the blood. The concentration of fibrinogen in preserved blood also decreases in proportion to storage of the latter (F. R. Vinograd-Finkel'; A. N. Filatov and M. F. Gepp). The majority of authors note that towards the thirtieth day of storage fibrinolytic activity of preserved blood considerably increases.

We have studied several indices of coagulation factors in blood with various times of storage, stabilized according to Central "Order of Lenin" Scientific Research Institute of Hematology and Blood Transfusion Solution 7[b].

For the research, 15-20 ml of blood were taken from ampoules directly before transfusion to the patient. After standing for 15-20 minutes the plasma was drawn off. In order to determine prothrombin consumption, with the purpose of neutralization of preservatives, 0.1 ml of a 10% calcium chloride solution was added to 1 ml of preserved blood. This test was studied one hour after coagulation of the blood. Attention was not focused on dilution of the plasma with preservative.

The number of thrombocytes in 1 mm^3 of blood was determined by the method of Fonio, retraction of plasmatic clots according to M. S. Machabeli, tolerance of plasma for heparin according to Zigg, recalcification time of plasma enriched with thrombocytes, prothrombin consumption in serum one hour after coagulation of the blood according to the method of M. F. Machabeli, prothrombin activity of blood according to the one step method of Kvik, fibrinogen level and fibrinolytic activity of blood according to Bidvell.

Eighty two studies of preserved blood were carried out with storage time from 1-10 days (28 ampoules), from 11-20 days (34 ampoules) and from 21-30 days (20 ampoules). The results obtained are presented in the table.

The number of thrombocytes in preserved blood decreased rapidly and by the tenth-twentieth day of storage, was 40-35%, and by the thirtieth day--15% of normal. Towards the end of the second week, the agglutinating ability of the thrombocytes had decreased.

Retraction of a plasmatic clot towards the tenth day of storage of preserved blood was within normal limits (0.1-0.2), and on the twentieth day the normal retraction of a clot was observed in only 30% of the cases, in 70% complete lysis of the clot was noted. After 30 days of storage of preserved blood retraction of a plasmatic clot did not set in.

The tolerance of the plasma to heparin remained stable and did not deviate from normal during the course of the entire storage period of the blood. The recalcification time of plasma of preserved blood enriched with thrombocytes increased 1-1/2 times (148 sec) towards the third week of storage, and towards the fifth week--2 times (190 sec).

Results of research on prothrombin consumption and theory indicate that thromboplastic activity of preserved blood is at the physiological level

independent of storage time. In preserved blood, stored for 30 days, the index of prothrombin consumption had a tendency to grow. Apparently, the increase in thromboplastic activity of preserved blood stored for long periods occurs as a result of discharge of erythrocytic thromboplastic factors into the plasma, whose existence have been indicated in works of B. I. Kuznik, V. G. Baludy, Mey and Hess and other authors.

Results of Research on Coagulation Factors in Preserved Blood as a Function of Storage Time

Criterion	Fresh Blood (control)	Storage period of preserved blood (in days)		
		1-10	11-20	21-30
Number of thrombocytes in 1 ml of blood (in thousands)	200-300	88	82	48
Agglutinating ability of thrombocytes	Expressed	Expressed	Weakly expressed	Absent
Index of retraction of plasmatic clot (in ml)	0.1-0.3	0.1	Lysis (70%) 0.1 (30%)	Absent
Tolerance of plasma for heparin (in min)	2.5-4	3	4	4
Recalcification time of plasma enriched with thrombocytes (in sec)	80-100	148	152	190
Prothrombin consumption (in sec)	35-80	67	76	95
Prothrombin index (in %)	94-110	62	54	44
Fibrinogen concentration (in mg %)	200-420	190	157	141
Fibrinolytic activity (in %)	3.1-15.8	18	27	35

The prothrombin level in preserved blood decreased with an increase in the storage time: for the tenth day by 20-30%, towards the twentieth day by 30-40%, and towards the thirtieth day--by 50-60%.

The fibrinogen concentration in the first 10 days of storage of the blood decreased to the lower limits of normal and subsequently was in the range 157 141 mg %. Fibrinolytic activity in the first 1-1/2-2 weeks of storage of the blood increased negligibly, but towards the thirtieth day it reached 30-35%. This may be explained by the effect of factors of disrupted erythrocytes, the number of which increases in proportion to the storage of blood (B. I. Kuznik and I. K. Slobozhankina).

Thus, the activity of the majority of coagulation factors in preserved blood decreased with an increase in the storage time, but fibrinolytic activity increases.

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THAWING OF FROZEN BONE MARROW AND WASHING OF GLYCERINE OF IT BEFORE TRANSPLANTATION

A. G. Fedotenkov, I. D. Shishkina, L. P. Ignasheva and G. N. Kurbanova

According to literature data (F. R. Vinograd-Finkel' et al, 1963; A. G. Fedotenkov; Lovelock; Smith and Sailes), during freezing of biological objects from -50 to -3° recrystallization occurs which leads to disruption of cells. Therefore, in order to accelerate passage through the zone of critical temperature, rapid thawing is used.

There are different opinions concerning regimes of thawing of bone marrow cells. Many investigators recommend rapid thawing of frozen bone marrow suspension in a water bath at a temperature of 37-40° and consider such a regime to be optimal (A. G. Fedotenkov et al; S. S. Lavrik; Ferrebee et al; Pegg and others). Several authors have obtained good results of retention of cells during slow thawing of bone marrow under room temperature conditions (Kurnick).

In the present work data are presented on a study of the effect of various regimes of thawing on the survival of bone marrow cells. Bone marrow of health persons (donors) and of animals (dogs) prepared in Central "Order of Lenin" Scientific Research Institute of Hematology and Blood Transfusion solution No. and frozen with 15% glycerine solution and 10% serum in an aluminum flat container with a corrugated and displaced surface, were subjected to investigation The volume of frozen bone marrow in all experiments was 100 ml. The freezing regime was also constant and insured the best retention of bone marrow cells (the temperature was reduced to -13° at the rate of 1 degree per minute, then to -196° at the rate of 10 degrees per minute). Prior to freezing a thermocouple was placed in the container with the bone marrow, which was joined to an electrode which was equilibrated with a recorder which registered temperature. On the basis of such a recording temperature curves were obtained both during freezing and during thawing of the bone marrow.

The preservation of cells of frozen bone marrow was determined by microscopic examination of the preparations, supervitally surrounded with eosin, with simultaneous calculation of the number of nucleous-containing cells.

Three thawing regimes were used: rapid--in a water bath at 39°, slow--at room temperature (18-20°), very slow--in a refrigerator (4°).

Rapid Thawing

Bone marrow frozen to -196° in an aluminum container (the thickness of the layer of bone marrow suspension is 1 cm) was transferred from liquid nitrogen into a bath with a volume of 20 liter with water temperature of 39°. For more rapid and uniform thawing the bone marrow container was subjected to constant shaking. Measurement of temperature with the help of a thermocouple showed that warming of 100 ml of bone marrow from -196 to 0° occurs within 50-60 seconds, and to +17-20°--within 2 minutes. In this process the temperature increase of the bone marrow from -196 to -50 to -60° occurs with a rate of 850-860° per minute; then this process goes considerably more slowly: for example, up to -20° the bone marrow is warmed with a speed of 120° per minute, the increase of temperature from -20° slows down still more, and in the interval from

13 to 11 and up to 0° occurs with a rate of not more than 20° per minute. In several experiments plateaus were noted on the thawing curve of the bone marrow, indicating that in the period of recrystallization the rate of thawing slows down.

As a result of experiments it has been established that the duration of thawing of 100 ml of bone marrow suspension (at a temperature of -196 to 0°) consists of about 1 minute; heating from 0 to +2-5°, within 4-10 seconds and from 5 to 20° within 1 minute.

Bone marrow cells subjected to thawing were preserved better when heated only up to 2-5°; further increase of temperature of the bone marrow suspension is accompanied by a loss of survival of its cells (Table 1).

Table 1

Percent of Preserved Bone Marrow Cells (Eosin Sample), Thawed up to 2-5 and 20-23°

Number of Experiment	Percent Preserved Cells		
	Prior to Freezing	After Thawing	
		Up to 2-5°	Up to 20-23°
1	95	85	65
2	93	88	70
3	96	90	68
4	95	91	65
5	93	87	66
6	96	90	78
7	94	89	69
8	93	85	70
9	95	86	78
10	98	88	72
Average Data	95	87.9	70.1

We recommend that bone marrow preserved by thawing which is intended for clinical use be thawed in a period of not more than 60-70 seconds up to a temperature of 2-5°. Calculation of the absolute number of surviving nucleous-containing elements showed that with rapid thawing of bone marrow 85-90% of the cells remain viable.

Slow Thawing

Containers with frozen bone marrow were removed from liquid nitrogen and placed in a vertical position so that their surfaces did not come in contact with other objects. The temperature of the surrounding air was 18-20°. With the help of a thermocouple connected with a potentiometer, temperature of the thawed suspension was measured.

Experiments showed that thawing of 100 ml of bone marrow suspension from -196 to 0° at room temperature requires 50-55 minutes. Heating of the

suspension from 0-2° takes another 2-3 minutes and a further increase in temperature of the bone marrow to 18-20° occurs after 4 hours. During such a regime of thawing about 50% of the cells of the thawed bone marrow proved to be nonviable.

Very Slow Thawing

Frozen bone marrow was transferred to a refrigerator with a temperature of 4°. Thawing of 100 ml bone marrow to 0° occurred over the course of 3 hours and subsequent heating up to 4° required another 4 hours.

During such very slow thawing of the bone marrow many cells were nonviable.

Washing of Glycerine from Thawed Bone Marrow

In order to maintain the thawed bone marrow in a biologically intact state the correct selection of a method of washing protective substances from it, in particular glycerine and products of cellular decomposition, is of essential importance.

The majority of investigators consider that it is undesirable to transplant thawed bone marrow in which a 15% glycerine solution was used for preservation without preliminary reduction of the concentration of the protective substance. Introduction of bone marrow cells from which the glycerine has not been washed into the blood stream of a recipient is accompanied by a significant osmotic lysis of these cells (Ferrebee et al, 1967, 1969, 1963; Newton et al, 1959 and others). In 1956 Sloviter proposed a method for reducing the glycerine concentration in thawed erythrocytes by means of dilution of a suspension of cells with solutions of varying osmotic concentrations. Subsequently this method was used with modifications for washing bone marrow (Ferrebee et al, 1957). According to the method of Sloviter, Ferrebee and other foreign investigators used highly concentrated solutions of glucose (35% or 33.5%) and isotonic salt solutions (Henks, Tirode, etc.) in order to wash protective agents from the cells. However, according to the data of several authors, the process of washing glycerine from bone marrow leads to a considerable loss of cells (Mannick et al; Smith; Witte).

Considering the importance of preparation of thawed bone marrow to transplantation, we focused great attention on questions of reduction of concentration of glycerine and cellular decomposition products in the bone marrow suspension. In 1960 a glucose-sucrose solution containing 48.6% glucose solution and 8% sucrose solution had already been developed in the laboratory of tissue preservation of the Central "Order of Lenin" Scientific Research Institute of Hematology and Blood Transfusions. We consider the addition of sucrose to the wash solution instead of salt components justified, since there are data in the literature indicating that nuclear cells (leucocytes) poorly transport isotonic solutions of sodium chloride (N. B. Chernyak, 1957). Sucrose, as was revealed during preservation of blood, proved to have a marked stabilizing effect on protein systems (P. S. Basil'yev and N. P. Karaseva, 1946, 1952), which gives the structure of the cells resistance to disruption.

During the development of a method of washing glycerine from large bone marrow samples with the help of glucose-sucrose solution, good results were

obtained regarding the preservation of bone marrow cells of both animal (dogs) and people. For comparison of the effectiveness of glucose-sucrose solution with salts, experiments were carried out in which thawed bone marrow was washed with glucose-salt solution No. 1, glucose-salt solution No. 2 and Central "Order of Lenin" Scientific Research Institute of Hematology and Blood Transfusion glucose-sucrose solution No. 3. In all three solutions the first part contained 48.6% glucose solution, but in the first two it was prepared in Henks' solution. The second part of solutions No. 1 and 2 consisted of Henks' salt solution, but in solution No. 2 the disodium salt of tetraacetic acid (EDTA Na_2) was added to it.

As results of the research showed, with the help of supervital eosin dye and calculation of the number of nucleous-containing cells the 5% of intact bone marrow cells is found after washing of thawed bone marrow with glucose-sucrose solution. The loss of cells in these experiments was not more than 6%, while when washing with glucose-salt solution about 23% of the cells were destroyed (Table 2). These data have been confirmed by study of the ultrastructure of the cells carried out in collaboration with Professor E. I. Terent'yeva.

Table 2

Preservation of Bone Marrow Cells (Eosin Sample) Upon Removal of Glycerine with Glucose-Salt Solution No. 2 and Central Order of Lenin Scientific Research Institute of Hemotology and Blood Transfusion Glucose-Solution No. 3

	Percent Preserved Cells			
	M	M±m	σ	P
Without washing	86.5	86.5±1.36	4.3	
Glucose-salt solution No. 2	63.1	63.1±2	6.3	0.001
Glucose-sucrose solution No. 3 Central Order of Lenin Scientific Research Institute of Hematology and Blood Transfusion	80.7	80.7±1.6	4.9	0.02

Thus, as a result of research carried out the following has been established. Rapid thawing in a water bath (at a temperature of 39°) is optimal for bone marrow cells. The best preservation of cells (in comparison with glucose-salt solution) is insured by glucose-sucrose solution, used for washing glycerine from the bone marrow.

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OBTAINING ALBUMINAG IN FRACTIONATION OF PLASMA BY COMBINED ALCOHOL-RIVANOL METHOD

L. V. Minakova and T. P. Khorobrykh

The author describes a method of preparing albumin preparation in fractionation of plasma by combined alcohol-rivanol method. The preparation was named albuminag; it contains 8 to 10% of plasma proteins, of which 70 to 80% is albumin, and the rest globulins. Albuminag is sterile, nontoxic and nonpyrogenic. Proteins of the preparation are protected from heat denaturalization by addition into albuminag of 15% glucose solution and 0.6% sodium caprylate.

Albumin in solutions of various concentrations (5-25%) are well recommended for treatment of traumatic, burn and operative shocks, chronic hypoproteinemia and hypoalbuminemia, determined by various principles (Kendrick; Janeway et al; Warren et al). According to effectiveness to them they do not yield preparations of albumin containing a certain amount of globulins. The process of obtaining them is more economical, since multistep purification of the preparations is eliminated.

Examples of such albumin preparations are the American preparation "plasmanite", containing 5% protein of which 80% is albumin, the Czechoslovakian one--a stable solution of plasma proteins, which is 65-85% albumin, and 4.5-5% protein, which is released by the Central Institute of Hematology and Blood Transfusion. Similar preparation permit fuller utilization of the plasma, to increase the yield of basic proteins and to reduce the cost of the preparation.

In order to dilute plasma proteins, and among other things in order to obtain albumin, Cohn proposed the alcohol method in 1946. Later the rivanol method began to be used for fractionation of plasma proteins (L. G. Bogomolova et al; N. M. Aleksandrova and Z. I. Novitskaya; Steinbuche and Niewiarowski; Miller and Copeland). In contrast to the alcohol method this method is simple, does not require complex equipment, low temperatures, insures a high yield of protein and, finally, may be combined successfully with other methods of fractionation.

In the Kirov Institute of Blood Transfusion the combined alcohol-rivanol method has been used since 1963 for fractionation of plasma with the purpose of obtaining fibrinogen, fibrinolysin and serum polyglobulin (L. V. Minakova et al, 1963, 1964). However, such valuable plasma fractions as albumin and a portion of the globulins, remain in the precipitate and are subsequently discarded.

With the aim of more complete utilization of the plasma fractions (primarily albumin), which were by-products during the preparation of the indicated preparations, we carried out the present studies.

After isolating fraction I from blood plasma according to Cohn, fibrinolysin according to the modified method of Shtepanek and a complex of immune

γ-and β-globulins according to the method of Tukachinskiy and coauthors the residual precipitate--a combination of serum proteins with rivanol--is a very viscous, difficultly separable mass of bright orange color. We tested several methods of dissolving the protein-rivanol complex and determined the conditions for extraction of albumin. Distilled water, acetate and phosphate buffer of various pH and several salt solutions were selected as solvents. The best solvent proved to be a solution of sodium chloride, which possesses the ability to bind rivanol with subsequent formations of an insoluble salt, which precipitates. The property of sodium chloride of entering into a reaction with rivanol and of forming an insoluble compound--acridine hydrochloride--is generally known (T. L. Senov). We have not encountered a report on the utilization of sodium chloride for removing rivanol from a protein-rivanol complex.

The highest protein content was obtained upon addition of a 0.9% solution of sodium chloride to the precipitate. With the help of electrophoretic analysis of the protein solution it was established that a great part of the protein must be in the albumin fraction.

The purpose of increasing the protein content in the solution in subsequent experiments the required ratio between the volume of protein precipitate and the sodium chloride solution was selected. The highest protein content was obtained at a ratio of isotonic sodium chloride solution and protein precipitate of 2:1. This mixture was carefully mixed and kept for not less than 10-20 hours at a temperature of 4 and 6° and at pH 5.4-5.8.

As a result of studies carried out conditions were found at which it is possible to separate proteins from the protein-rivanol complex, and a preparation was prepared containing 8-10% plasma proteins, of which 70-80% must be in the albumin fraction. The preparation is called albuminag, i.e., albumin + + α- and γ-globulin, since the basic mass of proteins must be in these two fractions.

In order to introduce the preparation into practice it was necessary to study the method of sterilization of the preparation in order to free it from epidemic hepatitis virus, to select stabilizing substances capable of preventing heat denaturation of the protein, to develop detailed technology in order to produce a sterile preparation, acceptable for therapeutic purposes.

It is a difficult problem to obtain albumin preparations free from epidemic hepatitis virus. The most reliable method of destroying the virus is thought to be 10 hour heating of the preparations at 60°. It is known that proteins are not insensitive to the heat effect and it causes partial or complete denaturation. The rate of denaturation of plasma proteins decreases in the presence of fatty acids, their salts and hydrocarbons (F. Gaurovits; Scatchard et al, etc.).

At the present time in the Soviet Union glucose is used for stabilization of preparations of pure albumin (G. Ya. Rozenberg et al, 1961, 1964, 1965), abroad--sodium salts of mandelic and caprylic acids are used (Ballou et al; Boyer et al; Cailar et al). Since albuminag is a complex of albumin with globulins, it was necessary to select a stabilizer to insure the stability of all of these protein components.

Indices of Albuminag Preparation Before and After Heat Treatment

Series of Experiment	Stabilizer	Stabilizer Concentration (in %)	Before Heating								After Heating							
			pH	Relative Viscosity	Total protein (in %)	Ratio of Protein Fractions (in %)					pH	Relative Viscosity	Total protein (in %)	Ratio of Protein Fractions (in %)				
						Albumins	Globulins							Albumins	Globulins			
							α_1	α_2	β	γ					α_1	α_2	β	γ
I	Sodium mendalate	0,6	7,4	1,7	9,9	73,6	4,8	9,6	7,8	4,2	7,6	6,6	9,8	76,4	Globulins 23,6			
II	» »	0,8	7,4	1,8	8,6	73,2	3	10,2	9,2	4,4	7,5	6,0	8,4	78,8	» 21,2			
III	Sodium caprylate	0,6	7,5	1,8	9,0	74,6	3,2	11	7,0	4,2	7,2	4,9	9,0	78,0	» 22,0			
IV	» »	0,8	7,5	1,8	9,9	74	4,4	10,4	7,4	3,8	7,2	5,5	10,0	78,6	» 21,4			
III	Fructose	5	7,3	2,0	9,2	73	4	11	6	6	Samples coagulated upon heating							
	»	10	7,4	2,2	9,6	75	6	9	6	4	7,7	9,4	9,4	70	» 30,0			
	»	15	7,3	2,8	9,3	76	4	13	5	3	7,9	9,3	8,2	68	» 32,0			
IV	Sucrose	5	7,4	2,2	8,9	74	5	12	5	4	Samples coagulated upon heating							
	»	10	7,3	2,7	8,7	76	4	12	5	3	7,6	9,0	8,6	72	» 28,0			
	»	15	7,2	2,8	9,0	78	5	12	3	2	7,4	8,8	8,9	76	» 24,0			
V	Glucose	5	7,4	2,0	8,7	73	7	12	5	3	Samples coagulated upon heating							
	»	10	7,2	2,1	9,6	75,2	4,6	9,2	7,2	3,8	7,3	5,8	9,7	61	» 39,0			
	»	15	7,2	2,9	9,4	74,8	3,2	10,2	7,2	4,6	7,2	3,1	9,4	74,6	3,8	10,2	7,8	3,6
VI	Sodium caprylate	0,6	7,3	2,55	9,1	73	5	11	7	4	7,4	2,73	9,0	75	4	12	5	4

Note. Average data from 10 experiments of each series are presented in the table.

With this goal we set up five series of experiments. In Series I sodium mandelate in concentrations of 0.6 and 0.8% was used as the stabilizer of the proteins; in II--sodium caprylate was used in the same concentrations. In experiments of Series III-V the stabilizing effect of three hydrocarbons--glucose, fructose, sucrose, added to the protein solution at concentrations of 5, 10, and 15% were studied.

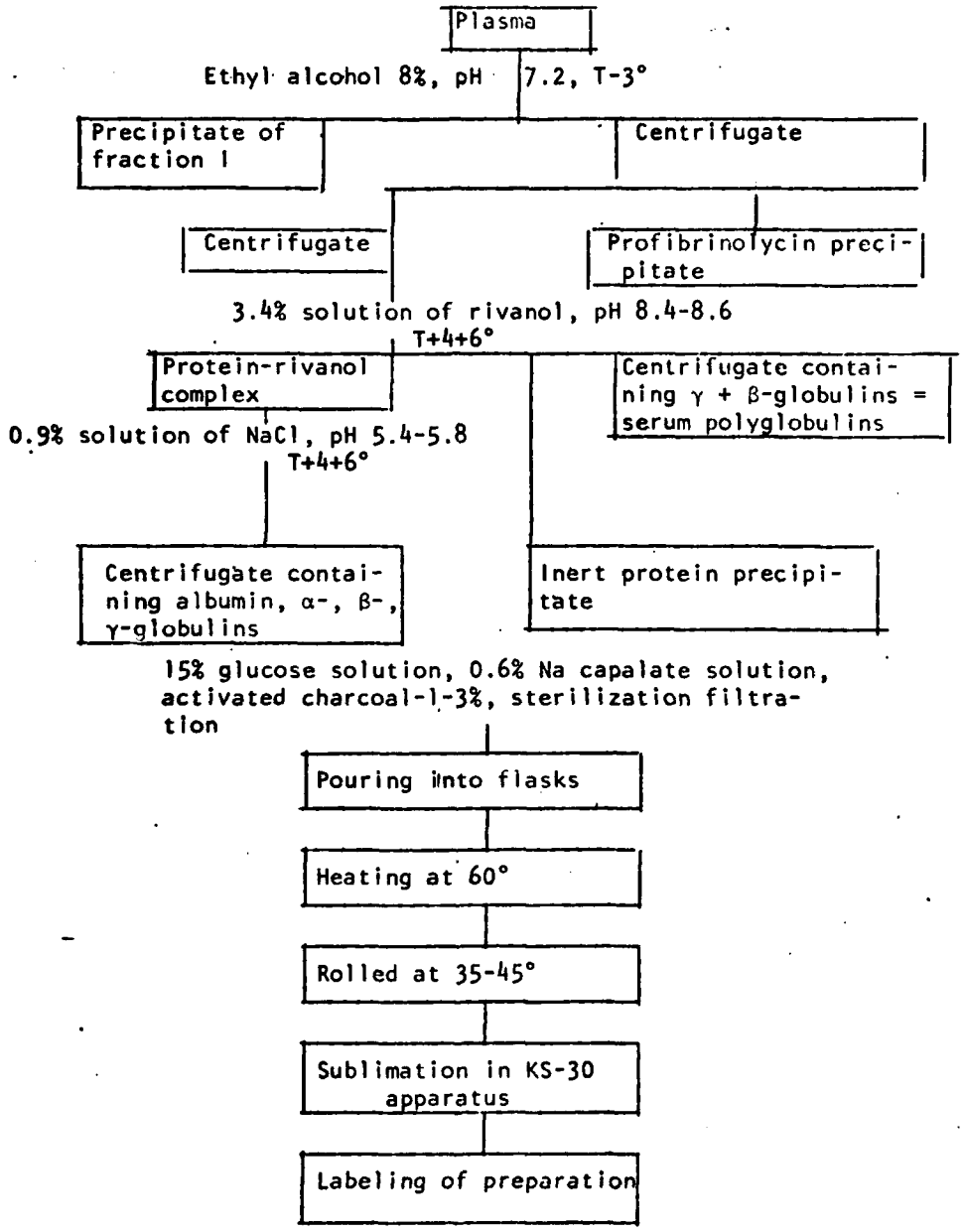
After addition of stabilizers the albuminag was heated in a water bath at 60° for 10 hours. In all the the series of experiments before and after heating the following were determined: 1) total protein by the biuret method with subsequent colorimetry of the samples in a photoelectrocolorimeter and calculation according to a calibration curve; 2) the ratio of protein fractions by the method of paper electrophoresis with subsequent densitometry; 3) index of acid-based equilibrium (pH) potentiometrically; 4) relative viscosity of these solutions with an Ostwald viscometer; 5) retention of native properties of isolated proteins by the method of immunoelectrophoresis in agar.

The research carried showed that sodium salts of mandelic and caprylic acids at both concentrations do not insure complete stability of the protein components of the preparation: after heating the solutions had considerable turbidity, increased their relative viscosity (Table 1). The electrophoreto gram had a very characteristic form: two spots appeared, one of which corresponded to albumin; it was not possible to separate the second one in individual fractions. The change revealed indicated the specific degree of denaturation of the protein. Apparently, sodium mandelate and sodium caprylate did not insure the stability of the most thermolabile globuline fractions. Use of 5% concentrations of sugars (glucose, fructose and sucrose) did not give a positive effect: all samples coagulated as a result of heating. Upon increasing the hydrocarbon concentration to 10%, the stabilizing effect was enhanced, but insignificantly; the solutions after heating were turbid, the relative viscosity increased. Thus, in samples with a 10% concentration of fructose the relative viscosity increased four times, in samples with sucrose--three times, with glucose--2.7 times.

In subsequent experiments the sugar concentrations were increased to 15%, but only in samples with glucose was reliable protection of the proteins of the preparation from the denaturing effect of heating achieved; the pH after heating did not change; the relative viscosity increased negligibly (on the average by 0.2); the ratio of protein fractions remained close to the initial value. When using a 15% concentration of fructose and sucrose the albumin samples were turbid, the viscosity of the preparation increased 3-4 times, the ratio of protein fractions changed. Therefore, we have selected glucose at a 15% concentration as the stabilizer of the albuminag preparation. Fifty series of albuminags were prepared, samples of which were kept at a temperature of 18-20% in a place protected from light. Six-eight months after production, flocculation occurred in several series, apparently caused by destabilization of globulins. Moreover, the relative viscosity increased insignificantly (1.5 times); the remaining indices remained unchanged.

By calculating the increasing in stabilizing effect, we carried out Series VI of the experiments, having used glucose at a 15% concentration in combination with sodium caprylate at 0.6% concentration. After heating the pH of the preparation remained close to the original value; the relative viscosity increased

Scheme for Obtaining Albuminag Preparation



on the average by 0.18; the ratio of protein fractions was essentially unchanged.

Immunoelectrophoretic analysis of alumbinag subjected to 10 hour heating shows that as a result of the formation of a specific antigen + antibody in the agar, clear arcs of precipitation appeared, corresponding to the basic components of albuminag--albumin, α-, β- and γ-globulins. Thus, all proteins of the preparation, including the most thermolabile γ-globulin, were not denatured in this experiment (see Figure).

Immunoelectrophoretogram of Albuminag,
Subjected to 10 Hour Heating at 60°.

The preparation stabilized by glucose at a 15% concentration and by sodium caprylate at a 0.6% concentration remains stable for a period of a year.

Let us present the scheme of preparation of albuminag.

The preparation is released in liquid and dry form. Liquid albuminag is a slightly opalescent liquid of the color of plasma, which frosts strongly upon shaking. The dry preparation is a yellowish-free flowing powder, readily soluble in distilled water and in isotonic NaCl solution. After dissolving, its indices are not substantially different from those of the liquid series. In Table 2 basic data of ten series of albuminag are presented. All series of the preparation prove to be sterile, nontoxic and nonpyrogenic. The yield of albumin was 70-80% in recalculation to albumin of the original plasma.

The albuminag preparation successfully passed experimental and clinical study and is recommended for wide application in therapeutic practice.

Table 2.

Basic Indices of Albuminag

Number of Series of Preparation	Total Protein (in %)	pH	Relative Viscosity	Ratio of Protein Fractions (in %)				
				Albumin	Globulins			
					α_1	α_2	β	γ
5	8,65	7,1	2,5	76	7	8	4	5
10	9,4	7,4	2,7	75	6	9	6	4
15	10,1	7,3	2,4	74	5	10	6	5
20	8,65	7,2	2,4	82	4	8	3	3
25	8,65	7,1	2,5	74	4	12	5	5
30	9,01	7,2	2,3	74	4	13	5	4
35	9,5	7,3	2,8	75	6	13	4	2
40	9,5	7,0	2,9	76	4	13	5	2
45	10,2	7,2	3,0	82	4	7	5	2
50	10,2	7,0	2,9	75	4	13	5	3
Average	9,4	7,2	2,6	76,3	4,8	10,6	4,8	3,5

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A NEW METHOD FOR ATTACHING VICTIMS TO STRETCHERS

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The removal of litter cases from inaccessible places (cellars, staircases of destroyed buildings and industrial structures, gullies with steep slopes, mine shafts) is a very tedious process. Until now this has been accomplished using stretcher straps and ropes. This method is inconvenient in many ways and causes the victim additional pain and suffering. It is more effective to remove wounded and injured individuals who have been put on a stretcher at the place where injury occurred; this involves solving the problem of finding a better and more reliable way to attach the victim to the stretcher.

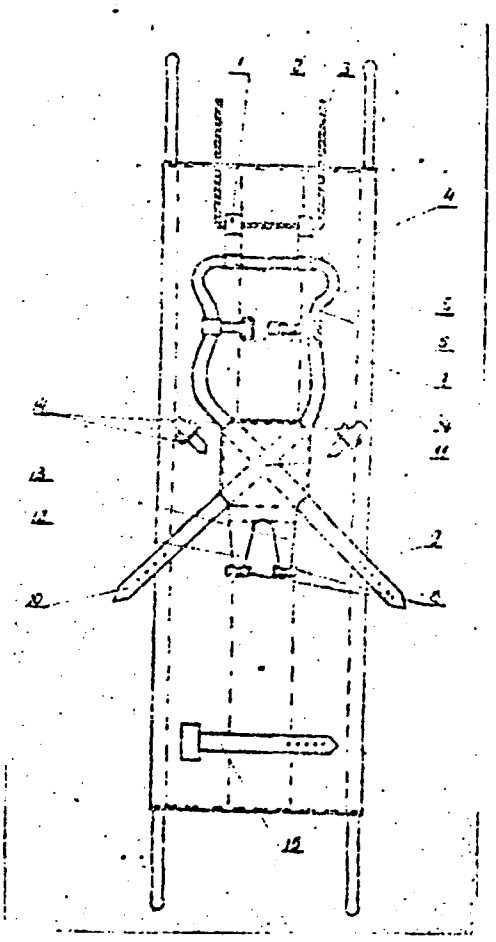

Figure 1. Attachment system in assembled form. Each component is identified in the text.

We have tested a modification to ordinary standard stretchers for attachment of victims which was proposed by M. A. Serebrov and M. I. Vlasov. It is superior to the ordinary method for attachment of wounded and injured persons to stretchers by straps or belts.

Essentially the modification is as follows. Additional pieces of material are sewn to the "bed" of the stretcher; these are of the same material or some other sufficiently strong fabric. Clasps are added. By means of these parts the freely moving straps and catches of the snap hook type are connected to the stretcher "bed".

The modification of standard stretchers is accomplished in the following way (Figure 1). Two straps are sewn to the head of the stretcher "bed". These straps have loops ("tunnels"): upper back (1) and (2). These are for passing through a rope (3) in cases when the victim must be transported in a vertical position. There are also lower back loops (4) and (5). An X-shaped "tunnel" (11) with freely hanging flaps (12) and (13) under the buttocks and buckles (8) is sewn at the pelvis level. Clasps (14) are sewn to the "bed" of the stretcher at the level of the upper edge of the cross piece. A strap (15) is sewn at the lower edge of the stretcher "bed" for holding the shin.

A freely moving main circular strap (6) is attached to these sewn parts. This requires that the free end (9) of this strap be pulled in an oblique direction through the X-shaped "tunnel" (11) from bottom to top, from left to right; then the snap (7) is attached. Then the end of the strap (9) is pulled through the lower back loops (5) and (4), after which a part (7a) is snapped on. Then the free end (9) is pulled through the X-shaped "tunnel" from top to bottom, from left to right, and hangs freely, similar to the free end (10) of the main circular strap.

The victim is secured by clasping four connections. This requires that the fastening (7) be clasped to the ring of part (7a) at the level of the middle of the breastbone, after which the ends of (9) and (10) are drawn tight. The end of (10) is passed over the right hip and secured with the clasp (8), sewn to the flap of the corresponding side (12) whereas the end of (9) is clasped in a similar way to the left side. The free ends (9) and (10) are secured by clasps (8) with a tension which does not press too hard against the hip. The shins are held with a strap (15). After clasping the mentioned straps, the chest part of the main circular strap holds the chest against the stretcher bed and loops are formed in the armpits by means of which the backbone is relieved of strain when the victim is transported in a vertical position. The transport of the victim in a vertical position is favored by the fact that by means of the ends (9) and (10) of the circular strap, clasped to the flaps (12) and (13), a seat is created beneath the buttocks on which the victim is supported.

Since the main circular strap moves freely, when the stretcher with the victim is moved in a vertical position there is a uniform distribution of the victim's body weight in different parts of the entire system. Individaul parts of the victim's body (chest, hips) therefore are not subjected to pressure. Moreover, the entire weight of the victim is distributed on the rope, on the straps with loops, on the main circular strap and on the flaps beneath the buttocks. For this reason the stretcher "bed" does not experience the load of the victim's body weight and there need be no fear that it will break when the victim is transported in a vertical position. The attachment components must have the necessary strength.

If the victim has a fracture of one of the hips (or shins) a special splint is applied to the fractured leg. However, in this case the free end [(9) or (10)] of the main circular strap is not passed over the hip of the fractured leg but over the pelvis and is secured with the clasp (14) on the opposite side.

When the transported victum is secured in this way he can be carried in a horizontal or vertical position (Figure 2) or with the stretcher turned on its side.

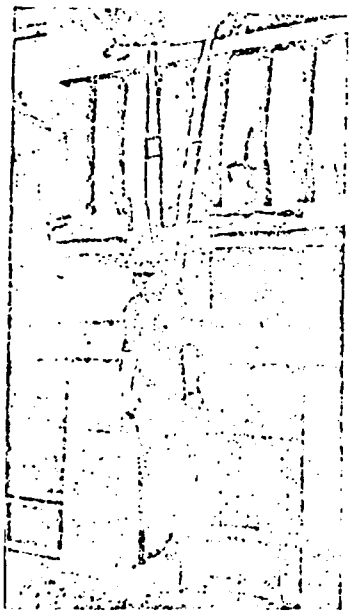

Figure 2. A victim being evacuated on a stretcher from the second story of a ruined building.

If both legs are fractured they are put into transport splints. The free ends are passed across the victim's pelvis in the front in a criss-cross fashion and the clasps (14) on the opposite side are attached. The securing of the victim in this way makes it possible to carry the victim through narrow passageways with turning of the stretcher by 90°; transport in a vertical position is possible only at a low height and when there is no injury to the chest and shoulder girdle.

In the case of fractures of the upper extremities they are immobilized by transport splints and the victim is then secured to the stretcher in the usual way.

In case of necessity splints are inserted into the pockets sewn to the rear surface of the stretcher "bed" in order to give it rigidity. This makes it possible to transport a victim with an injured spine in a horizontal or vertical position and "sideways" in narrow passageways.

This modification does not have special straps for holding the arms and head. In case of necessity the head can be held by round passes of a bandage passing through the loops (1) and (2) and for holding the arms it is sufficient to secure them with a bandage at the level of the wrist and tie them to the end of the main circular strap (9) or (10) already attached across the hip or pelvis. However, this need be done in but few cases.

Among the positive qualities of this particular modification we can also note that it is general purpose, suitable for persons of any height, because the victim is secured by the freely moving main circular strap and a snap clasp. It is particularly important that the victim need not be shifted from one stretcher to another because standard stretchers are suited for being transported in any type of vehicle.

Our tests revealed that this stretcher modification affords the possibility for securing to a stretcher a victim clad in outer clothing, wearing high boots, overcoat or sheepskin coat, because the presence of padding in the back on clothing of these types does not prevent clasping of the straps, something very important for winter transport.

The authors feel that the proposed modification of standard stretchers will find extensive use in mine shafts, open pit mines and quarries and will also be employed by emergency rescue crews and medical teams in the civil defense system. The described stretcher modification can even be fabricated from materials at hand with a minimum expenditure of time and material.

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